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Performance Characteristics of Different Immunoassays for Determination of Parathyrin (1–84) in Human Plasma Samples

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Summary: The performance characteristics of four radioisotopically and non-radioisotopically labelled two-site immunoassays for the determination of 'intact' parathyrin in plasma samples are reported.

Within-run as well as between-assay imprecision was characterized by coefficients of variation usually < 10%.

Assessment of the linearity of dilution in plasma samples from patients with severe secondary hyperparathyroidism (obtained from patients with chronic renal failure prior to dialysis) revealed that an assay with N-terminal capture antibodies showed an increase of the values after dilution ($p < 0.05$) whereas another assay with C-terminal capture antibodies was characterized by a decrease of the values after dilution ($p < 0.05$).

Correlation between the data obtained by the four assays and our currently used routine method (N-tact® PTH from INCSTAR) revealed correlation coefficients of $r > + 0.96$ and slope values between 0.83 and 1.34.

Determination of the analytical recovery of parathyrin (1–84) from two reference materials [First International Reference Preparation 79/500 and synthetic human parathyrin (1–84)] revealed that the recovery rates were strongly influenced by (a) the assay employed for determination of parathyrin concentrations, (b) the matrix of the diluent and (c) the reference material used. These results as well as systematic differences between the assays we examined (employing plasma samples from unselected nephrological patients) require further efforts towards a more rigorous standardization of 'intact' parathyrin assays.

Introduction

Parathyrin, which regulates the calcium ion concentrations in extracellular body fluids, is a single polypeptide chain consisting of 84 amino acids (M_r = 9425). The hormone is metabolized within the parathyroid glands as well as by other organs (e. g. liver and kidney) with the cleavage site in the 33–41 amino acid region yielding N-terminal and C-terminal parathyrin fragments (for review see l. c. (1)).

Radioimmunoassays based upon antisera with specificity for these fragments were characterized by the following disadvantages:

(a) clinically unacceptable lower detection limit,

(b) discordant results and

(c) reduced clearance of immunoreactive C-terminal parathyrin-fragments in patients with renal insufficiency due to its longer biological half-life as compared with the 'intact' molecule (2).

To overcome these disadvantages methods have been developed in recent years for specific determination of parathyrin (1–84) ('intact parathyrin'). These include

(a) assays employing antisera against parathyrin (24–48) (i. e. the proteolytic region of parathyrin) (2),

(b) sequential extraction of N-terminal immunoreactivity with subsequent midregion(44–68)-specific radioimmunoassay (3) and

(c) two-site immunometric assays employing two different antibodies (either monoclonal or polyclonal) with N-terminal as well as midregion/C-terminal specificity (4–9). Here we report on the performance characteristics of different radioisotopically and non-radioisotopically labelled immunometric assays for specific determination of 'intact' parathyrin with special regard to samples from unselected nephrological patients.

Materials and Methods

Samples

Venous blood specimens (taken between 8 and 9 a. m. and anticoagulated with the dipotassium salt of ethylenediaminetetraacetic acid) were obtained from unselected nephrological patients. The plasma fractions were separated from the blood cells within one hour after specimen collection. In order to avoid repeated freeze-thaw cycles at least two aliquots from each plasma sample were stored at −20 °C (for a period of up to 6 months). All analyses were performed immediately after thawing at + 4 °C.

Methods for determination of human parathyrin (1–84)

Four methods for specific determination of human parathyrin (1–84) concentrations were examined:

Method A
Immulite® Intact PTH (DPC® Diagnostic Products Corporation, Los Angeles, CA, U. S. A.)

Method B
PTH-EASIA (no. 40148 00; Medgenix Diagnostics SA, Fleurus, Belgium)

Method C
Intact-PTH-IRMA (no. 11041; Immuno Biological Laboratories, Hamburg, Germany)

Method D
Gamma BCT Intact PTH IRMA (no. AA-52F1; Immunodiagnostic Systems, Boldon, U. K.)

For method comparisons, the procedure we currently use for determination of parathyrin (1–84) was employed (N-tact® PTH; no. 22800; INCSTAR Corporation, Stillwater, MN, U. S. A.) (reference interval [2.5th–97.5th percentile; age-range: 20–60 years]: 1.1–5.8 pmol/l).

All kits examined are heterogeneous immunoassays of the immunometric type and were manually performed in duplicates according to the manufacturer's recommendations.

Calibration of kit A is based upon a lot-specific 'master curve' (as assessed by the manufacturer) with a re-calibration procedure repeated every two weeks employing 'Intact PTH Adjustors (low/high)'. Calibration of kit B was performed by linear interpolation between the plotted analytical results of the standards using Behring ELISA processor III (Behringwerke AG, Marburg, Germany). For kits C and D, respectively, a standard curve was constructed by a 'smoothed spline' algorithm (using a 1470 Wallac Wizard™ Gamma Counter).

More details concerning the kits are listed in table 1.

Reference materials

The following reference materials were employed:

(a) First International Reference Preparation of Parathyroid Hormone, Human, for Immunoassay (1st IRP 79/500; National Insti-

Tab. 1 Principles of the parathyrin (1–84) tests performed.

Method	Source and specificity		Label	Solid phase	Reagent volume [µl]		Assay incubation	
	Antibody bound to the solid phase	Labelled antibody			Sample	Labelled antibody	Time[4] [h]	Temperature [°C]
A	Goat anti-parathyrin (44–84)	Goat anti-parathyrin (1–34)	Alkaline phosphatase[1]	Bead	50	50	1	37
B	Goat anti-parathyrin (1–34)	Mouse anti-parathyrin (44–68)	Horseradish peroxidase[2]	Microwell	200	50	2 + 0.5	22
C	Sheep anti-parathyrin (39–84)	Sheep anti-parathyrin (1–34)	^{125}I	Tube	200	50	22	22
D	Mouse anti-parathyrin (1–34)	Goat anti-parathyrin (39–84)	^{125}I	Tube	200	200	4 + 18	4
E[3]	Goat anti-parathyrin (39–84)	Goat anti-parathyrin (1–34)	^{125}I	Bead	200	100	22	22

[1] with chemiluminescent substrate (a phosphate ester of adamantyl dioxetane)
[2] with chromogenic substrate (tetramethylbenzidine)
[3] N-tact® PTH (employed for comparison studies)
[4] methods B and D are "two-step-assays", the other "one-step-assays"

Tab. 2 Within-run imprecision of the parathyrin (1–84) methods examined (n = 10).

Method	Mean [pmol/l]	Standard deviation [pmol/l]	Coefficient of variation [%]
A	3.8	0.20	5.3
	86.1	5.51	6.4
B	3.9	0.35	9.0
	85.0	3.16	3.7
C	2.8	0.20	7.1
	19.7	0.39	2.0
D	4.6	0.23	5.0
	49.2	2.03	4.1

Tab. 3 Between-day imprecision of the parathyrin (1–84) methods examined (n = 8).

Method[1]	Mean [pmol/l]	Standard deviation [pmol/l]	Coefficient of variation [%]
A	4.8	0.33	6.9
	45.0	5.44	12.1
B	3.1	0.43	13.9
	39.7	3.24	8.2
C	2.9	0.19	6.6
	20.8	0.81	3.9
D	3.2	0.30	9.4
	16.3	1.98	12.1

[1] For assessment of between-day imprecision the quality control materials of the respective kits were used.

tute for Biological Standards and Control, London, U.K.) (assigned ampoule content: 0.1 IU/ampoule [corresponding to approximately 10 pmol parathyrin]) (purity > 95%) (10).

(b) Synthetic parathyrin (1–84) (human) (purity as assessed by HPLC > 99%) (no. H-1370; lot no. 504782; Bachem Feinchemikalien AG, Bubendorf, Switzerland) (assigned vial content: 0.5 mg parathyrin). Both reference materials are claimed to be free of parathyrin fragments by HPLC analysis.

Statistical Methods

The statistical methods employed include the *Passing & Bablok* (11) procedure for method comparison studies as well as *Friedman*'s test (employing the software package SPSS/PC + V2.0) for assessing differences between the parathyrin values at different dilution steps.

Results

Imprecision

Different human plasma pools were analysed 10 times to assess within-run imprecision of methods A, B and D, respectively. For method C 'controls 1 and 2' (components of the kit) were used (tab. 2).

The results of between-day imprecision are shown in table 3.

Lower limit of detection

Twenty replicate analyses were made within a single run employing

(a) the zero standards of the kits (methods B and C),

(b) parathyrin-free 'sample diluent' (component of the Immulite® Intact PTH kit) (method A and D).

The lower limit of detection, defined as the concentration corresponding to the 95% confidence interval of counts/absorbance, was found to be 0.1 pmol/l (methods A and B) and 0.2 pmol/l (methods C and D).

Linearity of dilution

Linearity of dilution was investigated using plasma samples from patients with chronic renal failure and severe secondary hyperparathyroidism, immediately before dialysis (range of parathyrin values: 59–206 pmol/l) employing the following diluents:

(a) Parathyrin-free 'sample diluent' (component of the Immulite® Intact PTH kit) (method A),

(b) the zero standards of the kits (method B and C) and

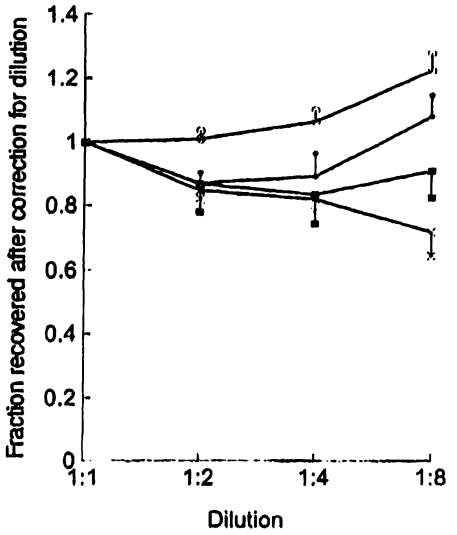

Fig. 1 Linearity of dilution
Plasma samples (from patients with chronic renal failure and severe secondary hyperparathyroidism, immediately before dialysis) were mixed with different parathyrin-free diluents (for further details see under Results).
Different plasma samples were used for assays A–D.
The fractions recovered after correction for dilution were given as means ± SEM.
Assay A = dots (n = 5); assay B = circles (n = 5); assay C = crosses (n = 3); assay D = squares (n = 4).

(c) pooled serum after accelerated thermal degradation of parathyrin (parathyrin concentration < 0.1 pmol/l as assessed by the N-tact® PTH kit) (method D).

After correcting for the dilution factor and expressing the values as a fraction of the concentrations measured in the undiluted sample, the recovery rates (at a 1 : 8 dilution) ranged from 71 ± 5% (method C) to 122 ± 7% (method B) (mean ± SEM). For method B the values obtained in the diluted samples significantly increased compared with those in the undiluted samples ($p < 0.05$) whereas for method C the values obtained in the diluted samples significantly decreased ($p < 0.05$). For further details see figure 1.

Method comparison

Comparisons were made between methods A–D and N-tact® PTH (see Materials and Methods). The relationship between the values was characterized by linear regression coefficients between + 0.968 and + 0.987 (fig. 2).

Analytical recovery of parathyrin (1–84) from reference materials dissolved in different diluents

Lyophilized parathyrin preparations (1st International Reference Preparation 79/500 as well as synthetic human parathyrin (1–84)) were re-solubilized with a buffer solution (10 mmol/l sodium phosphate, pH 7.2, with 120 mmol/l NaCl and 2 g/l human albumin) yielding stock solutions of 2500 pmol/l [1st International Reference Preparation] and 2630 pmol/l [synthetic parathyrin (1–84) (human), respectively]. Stock aliquots were stored at −20 °C for no longer than 2 weeks. Immediately after thawing at + 4 °C stock aliquots were diluted 1 : 10, 1 : 20, 1 : 40, 1 : 80, respectively, with

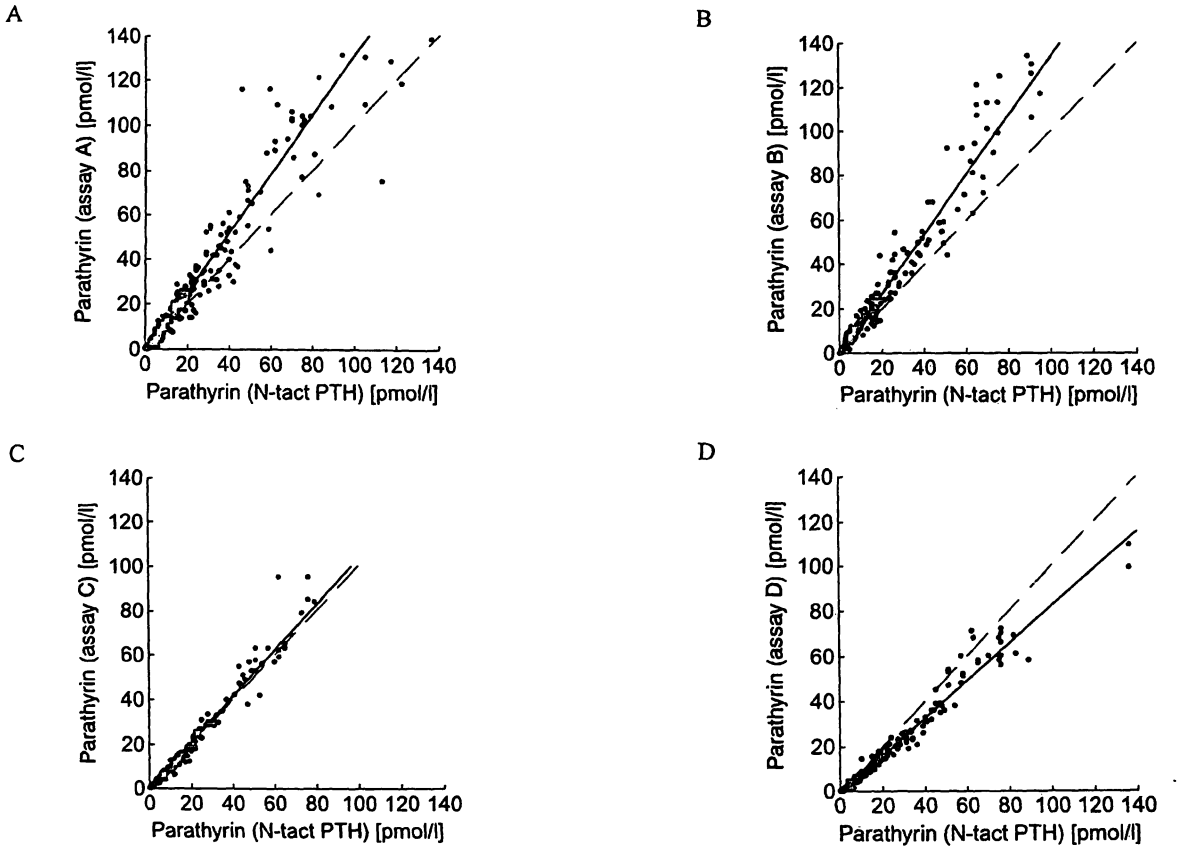

Fig. 2 Method comparison between methods A–D and N-tact® PTH (INCSTAR Corporation).
The following regression equations according to *Passing & Bablok* (11) were obtained:

Parathyrin [pmol/l] (method A)
= −1.00* + 1.32* × parathyrin (N-tact® PTH) [pmol/l]
(r = + 0.962; n = 394)

Parathyrin [pmol/l] (method B)
= + 0.01 + 1.34* × parathyrin (N-tact® PTH) [pmol/l]
(r = + 0.968; n = 277)

Parathyrin [pmol/l] (method C)
= −0.27* + 1.04* × parathyrin (N-tact® PTH) [pmol/l]
(r = + 0.987; n = 255)

Parathyrin [pmol/l] (method D)
= −0.54* + 0.83* × parathyrin (N-tact® PTH) [pmol/l]
(r = + 0.971; n = 279)

* significant deviation ($p < 0.05$) from 0 (y-intercept) and 1 (slope) according to *Passing & Bablok* (11).

(a) the zero standards of the methods employed,

(b) pooled serum after accelerated thermal degradation of parathyrin (parathyrin concentration < 0.1 pmol/l as assessed by the N-tact® PTH kit) and

(c) parathyrin-free 'sample diluent' (component of the Immulite® Intact PTH kit).

There was a strong dependence of the recovery rates upon

(a) the reference material used,

(b) the diluent and

(c) the method employed for determination of parathyrin (1−84) (tab. 4).

Discussion

Determination of the analytical recovery of parathyrin (1−84) from reference materials resulted in recovery rates of between 6% (!) and 75%. There is a marked dependence of the values obtained upon

(a) the matrix of the diluent,

(b) the reference preparations used and

(c) the assay employed.

A lower immunoreactivity of the First International Reference Preparation (No. 79/500) yielding recovery rates between 44% (4) and 60% (12) of those values obtained with chemically synthesized parathyrin (1−84) has already been described.

For the interpretation of the recovery rates of parathyrin (1−84) from the reference materials it has to be taken into account that

(a) the First International Reference Preparation 79/500 has not been chemically characterized and is therefore not suitable as a chemical standard but only for calibrating working standards for parathyrin immunoassays (10),

(b) the mass content of the ampoules coded 79/500 is based upon indirect estimates and claimed to be "approximately 10 pmol" (10) and

(c) no data are so far available regarding the immunoreactivity of the synthetic parathyrin (1−84) preparation employed.

Tab. 4 Analytical recovery of parathyrin (1−84) from reference materials dissolved in different diluents.

Method	Reference materials	Diluent[a]	Measured parathyrin concentration[b] [pmol/l]	Recovery rate[b] [%]	Recovery rate at further dilution steps[c] [%]
A	1st International Reference Preparation	Parathyrin-free 'sample diluent'	82	33	31 ± 2
	Synthetic human parathyrin (1−84)	Parathyrin-free 'sample diluent'	197	75	69 ± 1
B	1st International Reference Preparation	'Zero' standard	40	16	17 ± 2
	Synthetic parathyrin (1−84)	Parathyrin-free serum	111	42	42 ± 1
C	1st International Reference Preparation	'Zero' standard	14	6	5 ± 1
	Synthetic human parathyrin (1−84)	'Zero' standard	74	28	20 ± 1
		Parathyrin-free serum	125	48	43 ± 2
D	1st International Reference Preparation	Parathyrin-free serum	30	12	11 ± 1
	Synthetic human parathyrin (1−84)	Parathyrin-free serum	72	27	25 ± 1

[a] See Results for characterization of 'diluents'.
[b] At 1 : 10 dilution of stock aliquots corresponding to a calculated concentration of 250 pmol/l [1st International Reference Preparation 79/500] and 263 pmol/l [synthetic human parathyrin (1−84)]; for assessment of recovery rates the calculated concentrations were set as 100%.
[c] i.e. at 1 : 20, 1 : 40 and 1 : 80 dilution of stock aliquots (see above); means ± standard deviations of the recovery rates at these three dilution steps are given.

For the following reasons a loss of the polypeptide by surface adsorption or a degradation seems to be improbable:

(a) human albumin (free of protease activity) was added to the buffer solution used for reconstitution of the lyophilized parathyrin preparations (13); the National Institute for Biological Standards and Control claimed that a concentration of 2 g/l albumin in the buffer solution used for re-constitution of the reference preparation is sufficient (13);

(b) the re-solubilized reference materials were processed under optimal temperature conditions;

(c) the pH value in the buffer solution used for reconstitution was 7.4. *Schmidt-Gayk* et al. (14) prepared a similar standard solution of parathyrin (1−84) in a buffer solution adjusted to pH 7.4. The National Institute for Biological Standards and Control recommends a pH value of 8.6 (13).

The systematic differences between the methods we examined when using patient samples may be explained by the different specificity of the antibodies or differing assignments of standards against the reference preparations used by the manufacturers (5).

In patients with chronic renal insufficiency 'intact' parathyrin assays may be prone to a significantly negative interference by an excess of C-terminal fragments saturating the C-terminal capture antibodies (15, 16). All samples used to assess the linearity of dilution were obtained from patients with chronic renal failure and severe secondary hyperparathyroidism, immediately before dialysis. In these samples excessively increased concentrations of C-terminal parathyrin immunoreactivity can be observed (16). However, none of the methods examined in the present study with a C-terminal antibody attached to the solid phase (assays A and C) showed an increase of the recovery rates. On the contrary for method B (a two-step-assay with N-terminal capture antibodies) the values obtained in the diluted samples significantly increased compared with those in the undiluted samples ($p < 0.05$).

Within-run as well as between-assay imprecision was characterized by coefficients of variation usually being < 10%. This essentially corresponds to the values described earlier for two-site immunometric assays of 'intact' parathyrin (4−7).

Due to the highly significant correlation between the values obtained with different methods all assays may be equally useful for the diagnosis of secondary hyperparathyroidism in plasma samples from nephrological patients. However, to further improve the comparability of the values obtained with different methods as well as to minimize matrix effects efforts towards a more rigorous standardization (concerning the specificity of the antibodies as well as the assignment of the standards) should be made.

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